



# Expansion of the assembly of cowpea chlorotic mottle virus towards non-native and physiological conditions



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## ABSTRACT

The cowpea chlorotic mottle virus (CCMV) is a nanoparticle that holds promise for diagnostic and therapeutic applications. The empty virus-like particle, however, is not stable under physiological conditions. Here, we describe a systematic study into the expansion of the assembly properties of a protein-based block copolymer of the CCMV capsid protein and an elastin-like polypeptide. By systematically changing the hydrophobicity of the stimulus-responsive elastin-like polypeptide block, assembly of the capsid proteins could be achieved at close to physiological conditions. This strategy may prove to be useful in the development of a physiologically stable CCMV capsid variant.

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## 1. Introduction

Nanoparticles are important tools in the development of diagnostic and therapeutic tests for applications in medicine. In particular, virus-like particles (VLPs), i.e. viruses without their endogenous genetic material, are interesting scaffolds.<sup>1</sup> They are biocompatible, as their building blocks are proteins, and monodisperse and well-defined, due to the fact that they are always built up from a specific number of capsid proteins. *In vivo* studies on their use as drug carriers and other applications have already been performed, e.g. with the M13 bacteriophage, bacteriophage P22, tobacco mosaic virus (TMV), potato virus X (PVX), and cowpea mosaic virus (CPMV).<sup>2–5</sup> Even though these types of VLPs show great promise as imaging agents and drug delivery vehicles, they lack flexibility with regard to their encapsulation and modification properties, as they cannot be reversibly assembled and disassembled. The capsid proteins of the cowpea chlorotic mottle virus (CCMV), on the other hand, are able to reversibly assemble and disassemble, without their genetic material being present. The CCMV VLP assembles when the pH of the environment is lowered to 5.0. This results in icosahedral capsids with  $T = 3$  symmetry and inner and outer diameters of 18 and 28 nm, respectively.<sup>6,7</sup> When

the pH is increased to 7.5, the capsids fall apart into capsid protein dimers. These dimers arise from interdimer interactions, involving the N- and C-termini of the capsid proteins.<sup>7</sup> The reversible assembly of CCMV capsids has previously been used to encapsulate different types of cargoes, such as MRI contrast agents and enzymes.<sup>8,9</sup>

Even though no toxicity has been observed after injection of CCMV particles in mice, the capsids have not been applied frequently for *in vivo* studies.<sup>10</sup> This is due to the fact that, in order for CCMV to be stable under physiological conditions, it has to be held together by a negatively charged cargo polymer, e.g. nucleic acids.<sup>10</sup> Since this restricts the incorporation of a cargo, and thus the application of CCMV in the medical field, we set out to improve the stability of the CCMV capsid. More specifically, we would like to expand the conditions under which empty CCMV VLPs can be formed towards physiological conditions.

Previously, we developed a protein-based block copolymer of the CCMV capsid protein and an elastin-like polypeptide (ELP).<sup>11</sup> Elastin-like polypeptides are stimulus-responsive compounds, derived from the naturally occurring, highly elastic protein elastin.<sup>12</sup> They consist of repeating Val-Pro-Gly-Xaa-Gly (VPGXP) pentapeptides, where the guest residue Xaa/X can be any natural amino acid except proline. ELPs can be reversibly switched from a water-soluble state to a hydrophobic, collapsed state, in response to changes in the environmental conditions, such as an increase in temperature or salt concentration. The guest residue furthermore

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plays an important role in the transition process; a more hydrophobic amino acid will shift the transition temperature ( $T_t$ ) to lower values, whereas a more hydrophilic moiety will increase the  $T_t$ . A short ELP, consisting of nine pentapeptide repeats, was attached to the N-termini of CCMV capsid proteins, which are located in close proximity to each other in the interior of the VLPs. By introducing the ELPs, a new assembly pathway towards CCMV capsids was introduced.<sup>11</sup>  $T = 3$  VLPs could still be formed using pH-induced assembly, whereas the new ELP-induced assembly pathway resulted in smaller particles with  $T = 1$  icosahedral symmetry. The latter pathway could be initiated by an increase in the environmental temperature or salt concentration, leading to a higher local concentration of ELPs and subsequent assembly of the capsid proteins towards ELP-CCMV VLPs.

The development of the ELP-CCMV construct improved the assembly properties of the CCMV capsid protein such, that capsid formation could be induced at pH 7.5. Even though this is closer to physiologically relevant conditions, a high sodium chloride concentration (~2 M) was still needed to induce capsid formation. Here, we will introduce mutations in the guest residues of the short N-terminal ELP, in order to render this more hydrophobic. This should lead to ELP-CCMV constructs, which are able to assemble at lower salt concentrations at pH 7.5. In order to understand the effect of the mutations, these will be introduced in a systematic and controlled way. In this way, we hope to establish whether the switchable assembly behavior of the ELP-CCMV capsid proteins can be controlled by the hydrophobicity of the ELP and might even be predictable.

## 2. Results and discussion

In order to introduce more hydrophobic residues in the ELP of the ELP-CCMV construct, we started with the native ELP-CCMV construct: H<sub>6</sub>-ELP-CCMV( $\Delta$ N26). This construct has an N-terminal hexahistidine tag for purification purposes, followed by an ELP block, replacing the RNA binding domain of the wild-type CCMV capsid protein. The native ELP sequence is VPGVG-VPGLG-VPGLG-VPGLG-VPGLG-VPGLG-VPGLG-VPGLG-VPGLG, containing four valine guest residues, four leucine guest residues, and one glycine guest residue. In short, this ELP can be described using the general notation ELP[V<sub>4</sub>L<sub>4</sub>G<sub>1</sub>-9], where all guest residues and their ratios are given, followed by the total number of pentapeptide repeats.

We decided to target the valine and leucine residues and mutate them to either tyrosines or tryptophans to introduce more hydrophobic residues in the ELP (Table 1). A valine to tyrosine mutation can be done in the 1st position (first pentapeptide at the N-terminus), 3rd position, 5th position, and 8th position. We decided to compare a single mutation from valine to tyrosine on both the 1st and 8th position (VY1 and VY8, respectively), to investigate whether there would be a positional effect of the mutation.

**Table 1**  
Overview of the ELP-CCMV variants designed for this study, along with their calculated hydrophobicity factors.

Variant	ELP guest residues	Hydrophobicity factor <sup>13</sup>
Native	VLVLVLGV	19.9 °C
LY2	VYVLVLGV	11.0 °C
LY9	VLVLVLGVY	11.0 °C
VY1	YLVVLVLGV	8.7 °C
VY8	VLVLVLGYL	8.7 °C
LW2	VWVLVLGV	7.7 °C
VW1	WLVLVLGV	5.3 °C
VY1-VY8	YLVVLGYL	-2.6 °C
VW1-VW8	WLVLVLGW	-9.2 °C
VY1-VY5-VY8	YLVLYLGYL	-13.8 °C
VW1-VW5-VW8	WLVLWLGW	-23.8 °C

Additionally, two or three valine to tyrosine mutations could give information about the linearity of the effect of the hydrophobicity of the guest residues and the assembly behavior of the ELP-CCMV construct. To this end, we envisioned to make the VY1-VY8, and VY1-VY5-VY8 mutants. To see if valine to tryptophan mutations would have an even bigger effect, we also aimed at creating the VW1, VW1-VW8, and VW1-VW5-VW8 mutants. Lastly, to confirm the relationship between the hydrophobicity of the ELP and the assembly behavior of ELP-CCMV, we also envisioned to mutate leucine residues to more hydrophobic residues and compare these constructs to the above-mentioned ones. To this end, we designed LY2, LY9, and LW2 mutants.

In order to relate these ELPs in terms of hydrophobicity, we used a hydrophobicity scale developed by Urry.<sup>13</sup> Following this scale, transition temperatures of each possible guest residue in an ELP sequence were experimentally determined. Note that these  $T_t$  values highly depend on the salt concentration of the buffer and the concentration of the ELP itself. Nevertheless, we could use these values to calculate a hydrophobicity factor of all proposed ELP-CCMV mutants, by averaging the  $T_t$ 's of all nine guest residues (Table 1).

Next, we cloned all the designed constructs, starting from the original H<sub>6</sub>-ELP[V<sub>4</sub>L<sub>4</sub>G<sub>1</sub>-9]-CCMV( $\Delta$ N26) vector. We envisioned to use PCR driven mutagenesis to introduce the mutations in the 1st, 8th and 9th positions. All other guest residues could not be mutated using this strategy, as the required primers would have exceeded a length of 50 nucleotides. Not only would this have led to problems due to secondary structure formation in the primers, but also the chance of mismatches would have been high due the highly repetitive sequence of the ELP. Therefore, full-length DNA sequences were ordered for LY2, LW2, VY1-VY5-VY8, and VW1-VW5-VW8 and annealed in the desired vector. All other constructs were cloned, starting with PCR driven mutagenesis with the corresponding primers, and annealed into the expression vector (see SI for detailed experimental procedures).

After successful cloning of all ten ELP-CCMV mutants, we tested whether these variants could be expressed in *Escherichia coli* (*E. coli*). To this end, all vectors were transformed into BLR(DE3) pLysS cells and small scale test expressions were performed. Polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the lysates confirmed IPTG-induced expression of seven of the mutants; LW2, VY1-VY5-VY8, and VW1-VW5-VW8 had not been expressed (Fig. S1). Subsequently, the remaining seven variants and native ELP-CCMV were produced in a large scale expression, followed by purification via Ni<sup>2+</sup> affinity chromatography. SDS-PAGE and electrospray ionization time-of-flight (ESI-TOF) mass spectrometry analysis confirmed the successful expression of the proteins (see SI for experimental procedures and characterization of the expressed proteins).

In order to investigate the assembly behavior of the ELP-CCMV variants, we set out to determine the salt concentration at which each variant would start to assemble, at a certain concentration and temperature. In our studies, we chose to vary the concentration of sodium chloride and determine the transition NaCl concentration at certain temperatures.

For the first experiments, the fluorescent molecular probe 8-anilinoanthracene-1-sulfonic acid (ANS) was used as a read-out system for capsid assembly. The fluorescence intensity of this probe increases when it binds to hydrophobic regions of proteins. We used this dye to monitor the aggregation of the ELPs at certain NaCl concentrations. A sudden increase of fluorescence intensity could be linked to ELP aggregation, which is the driving force for capsid assembly. This assay was used to determine the transition NaCl concentration of native ELP-CCMV and the 7 variants thereof at four different temperatures (see SI for the detailed experimental

procedures). As a control, only buffer was added to the ANS dye; as expected, for all NaCl concentrations ranging from 0.15 to 2.0 M, the same emission intensity was measured in buffer (Fig. S2). For native ELP-CCMV, LY2, LY9, VY1, and VY9, a clear transition NaCl concentration could be observed, where the emission maximum stopped from being constant (Fig. S3). From this point on towards higher NaCl concentrations, a linear increase in the emission maximum was observed, corresponding to the continuous release of water from the aggregated ELP structures. When the data collected for each of these proteins at 20, 24, 28, and 32 °C are compared, a linear relationship between the transition NaCl concentration and the temperature was observed (Fig. 1A). When the same data was plotted slightly differently, it could be observed that there also seems to be a linear trend between the transition NaCl concentration and the hydrophobicity factor of the ELP (Fig. 1B). This indicates that the behavior of an ELP-CCMV construct is predictable, based on the hydrophobicity factor of the short N-terminal ELP. This data also shows that there is a slight difference in the behavior of LY2 and LY9, and of VY1 and VY8. In both cases, the mutation at the C-terminal end of the ELP seems to have the greatest effect on the assembly behavior of the ELP-CCMV construct. This might be explained by the fact that a mutation closer to the CCMV capsid protein will have more effect on its assembly. Furthermore, it can be noted that there seems to be no clear difference between a mutation of a leucine or valine residue; the hydrophobicity factor of the ELP is most influential when it comes to the resulting assembly behavior of the ELP-CCMV variant.

The behavior of the three most hydrophobic ELP-CCMV variants, namely VW1, VY1-VY8 and VW1-VW8 could not be measured under these experimental conditions. Instead of a sharp transition in the fluorescence emission maximum, a very subtle increase in the emission was observed going from 150 mM NaCl to 2000 mM NaCl (Fig. S4). This indicates that these variants were already assembled at 150 mM NaCl and that an increase in the NaCl concentration in this case only leads to loss of water from the aggregated ELP core of the capsids.

In order to confirm that the increase in the emission maximum observed in the ANS assay corresponds to a transition of ELP-CCMV dimers to capsids, the assembly was further investigated using dynamic light scattering (DLS) (see SI for the detailed experimental procedures). In this case, DLS could be used to distinguish between capsid protein dimers, which normally show a peak around 8 nm in

DLS distribution plots, and capsids, which are normally observed around 20 nm in DLS distributions. Firstly, the assembly behavior of ELP-CCMV variant LY2 was fully characterized at 20 °C. In both the intensity distribution (Fig. S5) and the number distribution (Fig. 2 and Fig. S6), dimers were observed at NaCl concentrations below 1500 mM. At 1500 mM NaCl and higher, capsids were visible. This data therefore confirms that the ANS assay is indeed able to distinguish between capsid protein dimers and capsids.

When the DLS data were compared to the data obtained in the ANS assay, it was found that the transition NaCl concentrations observed in the DLS distribution plots were consistently higher than the transition NaCl concentrations determined by the ANS assay. For LY2, a transition NaCl concentration of 1.23 M NaCl was determined by the ANS assay at 20 °C, whereas capsids were only observed starting from 1.5 M in the DLS distribution plots. We believe that the ANS assay is more sensitive to changes in hydrophobicity and the subsequent onset of assembly. Furthermore, the experimental set-up of the ANS assay also allowed for more controlled experimental conditions, such as temperature, incubation times, and waiting times in between measurements than the DLS procedure. We therefore think that the ANS data are more reliable than the DLS data. The trends with both techniques, however, are similar.

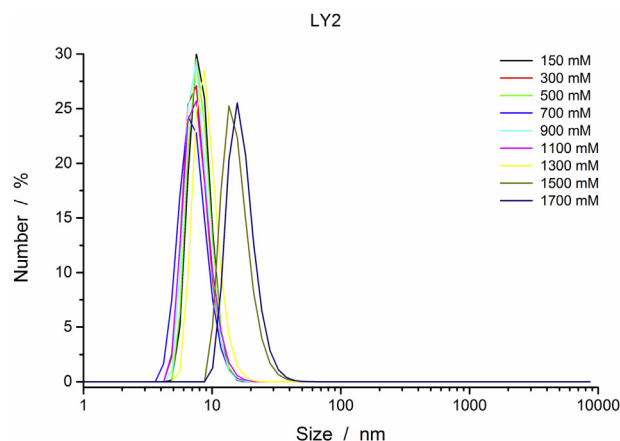


Fig. 2. Number distributions of LY2 ELP-CCMV at different NaCl concentrations, as determined by DLS.

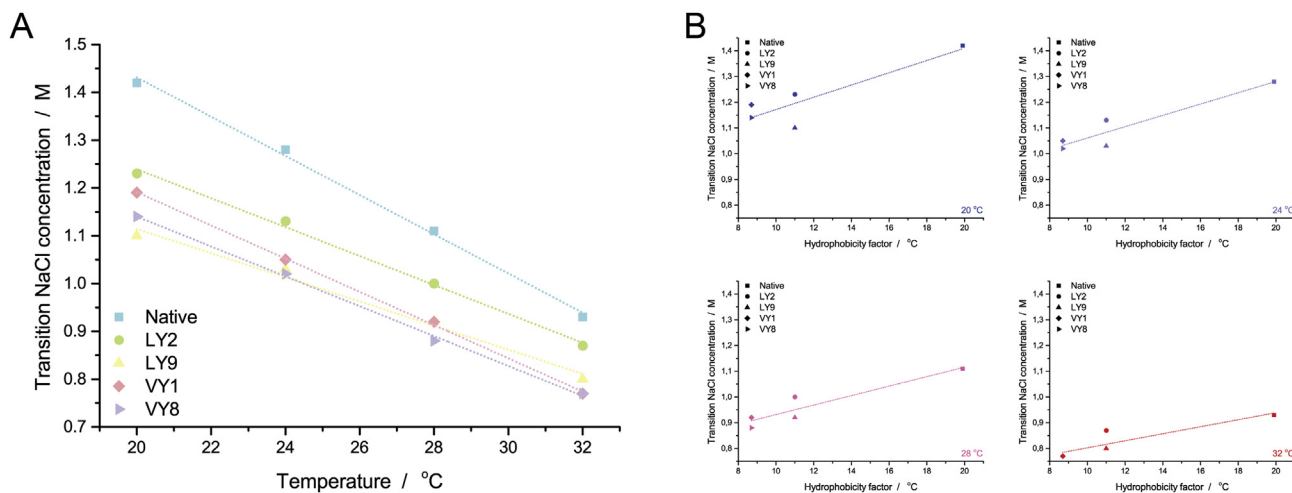
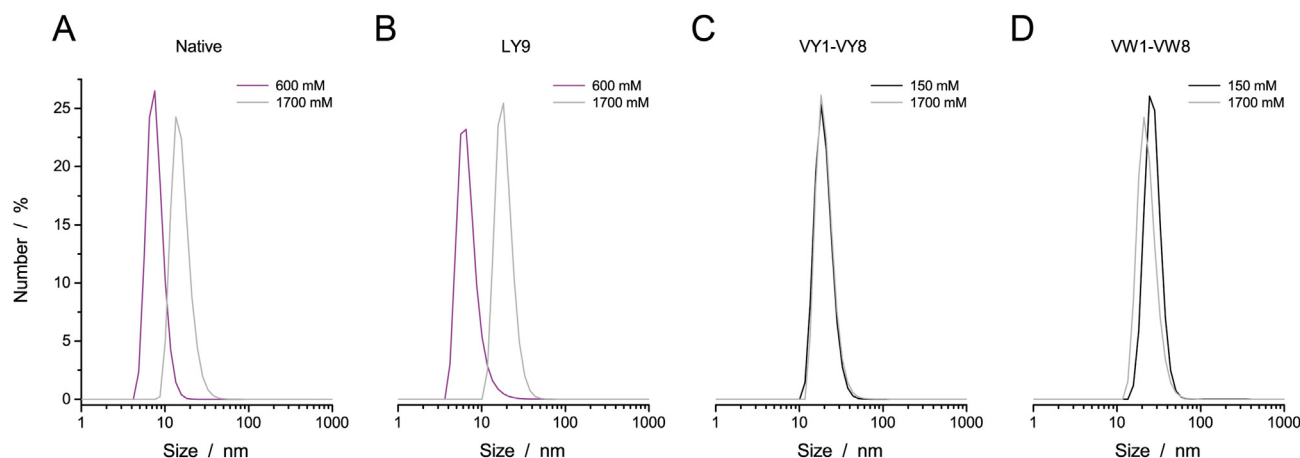


Fig. 1. Transition NaCl concentrations of native ELP-CCMV and four ELP-CCMV variants, measured using the ANS assay, at a final protein concentration of 20  $\mu$ M. A) Transition NaCl concentration plotted against the experimental temperature, per protein variant. B) Transition NaCl concentration plotted against the calculated hydrophobicity factor (Table 1), per experimental temperature. The linear fits were added to help visualize the observed trends.



**Fig. 3.** Number distributions at 600 mM or 150 mM and 1700 mM NaCl, as determined by DLS, at a final protein concentration of 20  $\mu$ M, of A) native ELP-CCMV, B) LY9 ELP-CCMV, C) VY1-VY8 ELP-CCMV and D) VW1-VW8 ELP-CCMV.

Next, we also checked the behavior of native ELP-CCMV and variants LY9, VY1-VY8, and VW1-VW8 using DLS (Fig. 3 and Fig. S7). As expected, we observed a transition from dimers to capsids for native and LY9 ELP-CCMV, going from 600 mM NaCl to 1700 mM NaCl. For VY1-VY8 and VW1-VW8, we did not observe any transition in the ANS assay, which led us to suggest that these proteins might be in the capsid state at all NaCl concentrations that were tested. This was indeed confirmed by the DLS data measured at 150 mM and 1700 mM NaCl. For both these proteins, only capsids and no dimers were observed at these NaCl concentrations.

### 3. Conclusions

We have described a systematic study into the effect of the guest residues in the short N-terminal ELP sequence, attached to the CCMV capsid protein. We found that the assembly behavior of the capsid protein depends in a predictable, linear way on the hydrophobicity of the ELP. ELP-CCMV variants were developed which could assemble at much lower NaCl concentrations than the native ELP-CCMV capsid protein. These results allow the construction of ELP-CCMV proteins which have the desired switchable assembly behavior. In our lab, studies are ongoing to find out whether the systematic variations in the ELP-CCMV construct can be applied to produce physiologically stable ELP-CCMV capsids.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.tet.2017.04.038>.

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